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The Cytoplasmic and Transmembrane Domains of MHC Class II β Chains Deliver Distinct Signals Required for MHC Class II–Mediated B Cell Activation

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Summary

Class II–mediated signals play potential roles in B cell activation and antigen presentation. The regions of the class II molecule participating in B cell signaling are incompletely defined. Our prior analysis of structural requirements of the cytoplasmic domain of A_β revealed that only the eight membrane-proximal residues are required for signaling. Here, we report that the sequence and position of two of these are critical, and present direct evidence that the A_β transmembrane domain is also involved in signaling, via a pathway distinct from the cytoplasmic domain. These results demonstrate that specific regions in both the cytoplasmic and transmembrane domains of the class II molecule have distinct signaling functions.

Introduction

Major histocompatibility complex (MHC) class II molecules are expressed constitutively on B cells, monocytes, macrophages, and dendritic cells, and can be induced in other cell types following various activation signals. In T cell–B cell interactions, MHC class II serves to present peptide antigen to T helper (Th) cells and to deliver activation signals to the B cell. Involvement of MHC class II–mediated signals in B cell homotypic adhesion (Kansas and Tedder, 1991; Kansas et al., 1992), antigen presentation (Nabavi et al., 1989, 1992; St. Pierre and Watts, 1991), rescue from anergy (Bishop et al., 1993, 1994), and proliferation and differentiation (Palacios et al., 1983; Bishop and Haughton, 1986; Baluyut and Subbarao, 1988; Cambier and Lehmann, 1989; Takahama et al., 1989; Hamano et al., 1990; Lane et al., 1990) has been demonstrated for both mouse and human B cells. Class II–induced increases in intracellular calcium, inositol phosphate turnover, and protein tyrosine kinase (PTK) activation have been demonstrated in human B cells (Lane et al., 1990; Mooney et al., 1990). In mouse B cells, cAMP generation is seen following MHC class II engagement and has been implicated in many of the processes attributed to MHC class II signaling (Wade et al., 1989; Bishop, 1991; St. Pierre and Watts, 1991; Fuleihan et al., 1992; Nabavi et al., 1992; Newell et al., 1993). A role for a serine/threonine kinase that translocates following MHC class II signaling has also been suggested (Cambier et al., 1987; Wade et al., 1989). Recently, evidence has been presented for elevation of intracellular calcium and PTK activation when MHC class II is cross-linked on a mouse B lymphoma, K46 (André et al., 1994), and we have confirmed this observation using normal splenic B cells (M. Baccam and G. A. B., unpublished data). Both we and others have also reported class II–mediated activation of proliferation of normal mouse splenic B cells (Baluyut and Subbarao, 1988; Cambier and Lehmann, 1989; Bishop et al., 1994).

Structure–function studies of MHC class II have demonstrated the importance of the cytoplasmic (CY) domain in various class II–mediated signals. These include nuclear translocation of a cytoplasmic cAMP-dependent kinase (Wade et al., 1989), and antigen presentation to a subset of Th cell hybridomas (Nabavi et al., 1989; St. Pierre and Watts, 1991). Our analyses of the mouse B cell clone CH12.LX indicate that only the eight membrane-proximal residues of the CY domain of the A_β chain are necessary for induction of class II–mediated antigen-dependent immunoglobulin M (IgM) secretion. Specific residues within this minimal CY domain are important to signaling, but complete removal of the A_β CY domain does not totally abrogate class II–mediated B cell activation (Harton and Bishop, 1993). Together with the recent report that class II molecules lacking CY domains are still capable of stimulating PTK activity in the K46 B cell line (André et al., 1994) these data suggest that other domains of the class II molecule also contribute to B cell signaling. The present report demonstrates that just two position-dependent amino acids in the A_β CY domains are critical to the contribution the CY domain makes to class II signaling, and that this contribution correlates directly with induction of elevated intracellular cAMP. Evidence is also presented that the transmembrane (TM) domain of A_β plays an essential role in class II signaling, similar in importance to that of the CY domain signal. However, signals delivered via the TM domain do not involve elevation of cAMP, and evidence is presented that the TM domain signals via a pathway distinct from that of the CY domain.

Results

Glycine 227 and Proline 228 Play a Dominant Sequence- and Position-Dependent Role in Class II–Mediated Signaling

In experiments examining signaling through A molecules with truncated β chains, we recently demonstrated that only the eight most membrane-proximal residues of the CY domain (amino acids 220–228; Figure 1A) are required for optimal levels of signal transduction (Harton and Bishop, 1993). Removal of proline (P) 228 or both glycine (G) 227 and proline 228 led to substantial decreases in class II signaling (mutants ABBΔ11 and ABBΔ12, respectively; Harton and Bishop, 1993; Figure 1A). A construct

A

Mutant	Amino Acid Sequence of CY (221 224 227 230 233 236)	Signaling (% of wt)
ABB(wt)	RHR SQK GPR GPP PAG LLQ	95
ABBΔ10	RHR SQK GP	96
ABBΔ11	RHR SQK G	31
ABBΔ12	RHR SQK	8
ABBΔ17	R	6
ABBΔ18		10
βCT1	RAA AAA AA	18
βCT2	RAA AAA GP	71
βCT4	RAG PAA AA	13
βCT5	RAA AAA AP	10
βCT6	RAA AAA GA	8
βCT7	RGP	5
G227V	RHR SQK VP	30
P228R	RHR SQK GR	40
P228Q	RHR SQK GQ	104

B

Diagram of transmembrane mutant	Signaling (% of wt)
	12

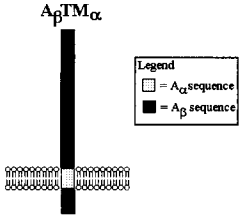
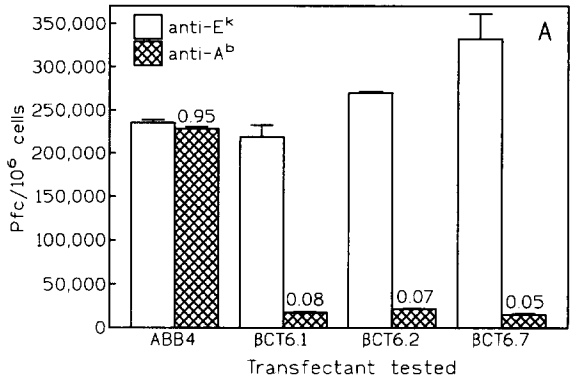


Figure 1. Structure of A_β^b Molecules with CY and TM Domain Mutations

(A) Amino acid sequence of CY domain truncation and replacement mutants and summary of signaling results for each group of transfectants. Ratio of signaling via A^b/E^k (%) was calculated as described in Experimental Procedures. No βCT3 mutant is listed because transfectants did not express βCT3 (RAAAGPAA) at acceptable levels for analysis.

(B) Schematic of the $A_\beta TM_\alpha$ molecule. The molecule has a wild-type CY domain, and the β chain TM domain has been replaced with that of A_α. Summary ratio of signaling via the transfectants expressing the $A_\beta TM_\alpha$ molecule versus wild-type endogenous class II is given at right.



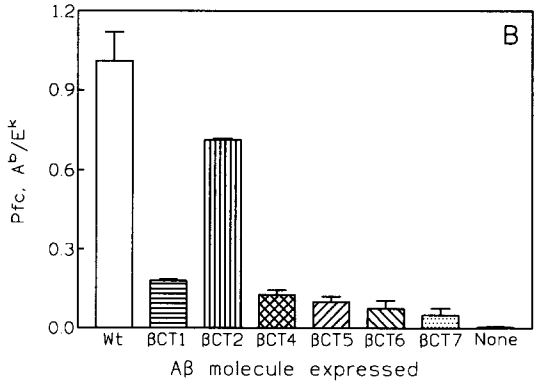


Figure 2. Class II–Mediated Signaling in Subclones of CH12.LX-Expressing A_β^b CY Replacement Mutants

(A) Example of how relative signaling is determined for each mutant A_β^b molecule produced. CH12.LX, the subclone ABB4 (expressing wild-type A_β^b), and the subclones βCT6.1, βCT6.2, and βCT6.7 (expressing the βCT6 mutant) were stimulated as described above. The number of pfc/10^6 viable cells was determined as described in Experimental Procedures. pfc values of control cultures (no stimulus) were subtracted from the values shown; control values were ≤2000 pfc/10^6 cells. Numbers above bars for each clone represent the ratio of the pfc values for cells cultured with Ag plus anti-A_β to the pfc values for cells cultured with Ag plus anti-E^k (calculated as described in Experimental Procedures).

(B) CH12.LX (none refers to the lack of A_β^b expression by CH12.LX, so the A_β^b-specific MAb does not signal), wild-type A_β^b, βCT1, βCT2, βCT4, βCT5, βCT6, and βCT7 transfectants were incubated with Ag (SRBC) alone (background values of <5%, which are subtracted prior to calculation of ratios) or Ag plus MAbs specific for endogenous E^k or transfected A_β^b molecules. pfcs were determined and the ratio of pfc to Ab/E^k was calculated as described in Experimental Procedures. Values are the mean ratios determined from 3–4 individual subclones.

replacing the minimal length cytoplasmic tail with a single arginine followed by 7 alanines (βCT1, Figure 1A) is incapable of restoring signaling, demonstrating that specific residues within the minimal CY domain are required (Harton and Bishop, 1993).

Because removal of G227 and P228 has such a profound effect on class II signaling, in the present study they were restored to their wild-type positions individually or together, or replaced in different positions in replacement CY domains similar to βCT1. The sequence of each of these replacement mutants is shown in Figure 1A. As is the case for all mutants analyzed (see Harton and Bishop, 1993), each of the βCT replacement mutants analyzed was expressed on the surface of individual transfectants of CH12.LX at levels equal to or greater than transfected wild-type A_β^b (data not shown). Although there is variation between subclones of a given transfectant in the degree of signaling through a single class II molecule, the ratio of signaling via a transfected molecule as a percentage of signaling through wild-type class II is a consistent measure of signal transduction. This is illustrated in Harton and Bishop (1993), and an example is shown in Figure 2A, in which three individual transfectants bearing the mutant βCT6 (discussed below) are analyzed. It can be seen that, although absolute numbers of IgM-secreting cells (plaque-forming cells [pfc]) differ for each transfectant, signaling induced via the βCT6 molecule compared with signaling induced via the endogenous E molecule (Figure 2A, ratios shown above cross-hatched bars) is similar between cell lines.

In the mutant βCT2 (see Figure 1A), G227 and P228 were replaced in their wild-type positions. βCT2 was able to signal at approximately 70% of wild-type levels (Figure 2B), indicating that G227 and P228 are crucial to signaling function. Having demonstrated with the βCT2 mutant that G227 and P228 were sufficient to restore class II–mediated signaling significantly, the ability of either G227 or P228 alone to mediate these effects was examined. The mutants βCT5, which replaces only P228, and βCT6, which replaces only G227 (see Figure 1A), do not signal above the level seen in βCT1 (Figure 2B). This demonstrates that neither G227 nor P228 alone is sufficient to restore signaling, and further suggests that the requirement for G at this position may be very stringent. To examine further the requirement for specific amino acids at positions 227 and 228, point mutants were selected from a library of point mutants (see Experimental Procedures) in the 8 aa truncated CY domain (Δ10). Two of these mutants, G227V and P228R, both show decreased signaling when compared with the wild-type A_β^b molecule, providing further evidence that G and P are specifically required at these positions. However, mutation to a residue found in human Eβ and DPβ CY domains (P228Q) allows wild-type levels of signaling (see Figures 1A and 4A).

That G227 and P228 in βCT2 are necessary and sufficient to restore the majority of the wild-type signal suggests that these residues may interact with an associated protein in a specific fashion. One possible type of interaction is a pocket, which can accomodate the fairly bulky P at position 228 provided that position 227 is not occupied by a residue more bulky than G. An interaction of this sort might impose positional dependence upon G and P. To address this possibility, we constructed βCT4 (see Figure 1A), which moves G and P together to a more membrane-proximal position. This molecule gives poor signals (Figure 2B), comparable to mutants lacking a CY domain (Δ18) and replacement mutants lacking G, P, or both (βCT1, βCT5, and βCT6). These results suggest that the position of G and P within the minimal length cytoplasmic tail is important for their ability to interact with intracellular signaling molecules. An additional construct, βCT7 (see Figure 1A), which contains G and P preceded by only the arginine anchor is also defective in mediating differentiative signals in CH12.LX (Figure 2B). Together, the data from these two mutants demonstrate that the function of G and P is dependent upon their position in the CY domain of A_β.

Glycine 227 and Proline 228 Are Sufficient for A_β-Mediated Increases in Intracellular cAMP following Class II Ligation

Previous studies demonstrate that class II–mediated signals to B cells are associated with elevated intracellular cAMP (Cambier et al., 1987; Takahama et al., 1989; Bishop, 1991), that inhibition of cAMP production inhibits class II–mediated signaling (Takahama et al., 1989; Bishop, 1991), and that the cAMP analog, dibutryl-cyclic-AMP (dbcAMP), can partially or totally mimic class II–mediated signals (depending upon the assay used to measure signaling) (Cambier et al., 1987; Bishop, 1991; St. Pierre and Watts, 1991; Nabavi et al., 1992). Thus, we predicted that the CY domain of A_β is required for the generation of the cAMP response. To test this prediction, we examined the ability of A molecules containing various mutations in the CY domain to deliver signals resulting in increased cAMP. Figure 3A shows that the minimal length CY domain capable of delivering wild-type signals as measured by induced IgM secretion (Δ10) is also sufficient for optimal cAMP generation. As for pfc assays, cAMP induction is measured for each transfectant by signaling through A_β^b compared with E^k; ratios are shown above bars and in Figure 3B. Removal of the entire CY domain abolishes cAMP generation in response to class II ligation (Δ18). However, the restoration of only G227 and P228 is sufficient to restore cAMP generation to levels indistinguishable from that induced via wild-type A_β^b (βCT2). This

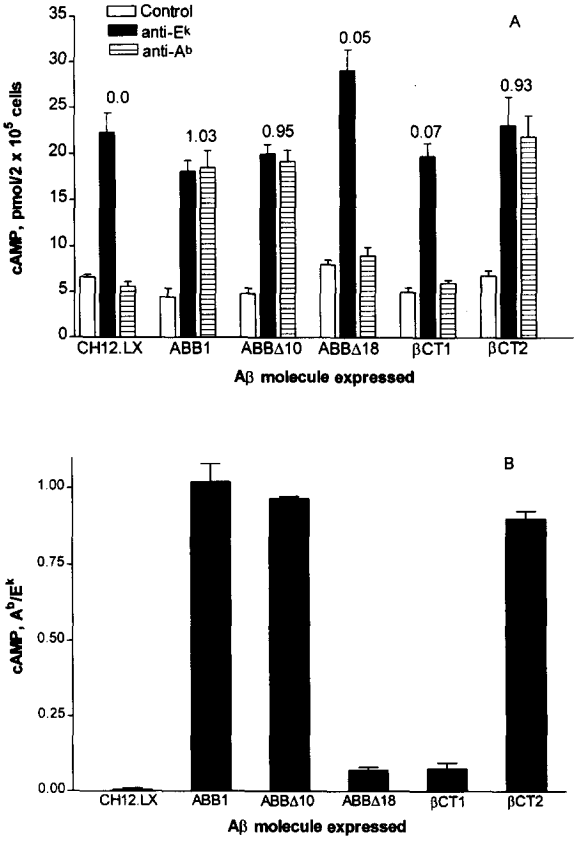


Figure 3. Class II–Mediated Generation of Intracellular cAMP in CH12.LX Cells Expressing wild-type A_β^b or CY Domain Mutants

(A) CH12.LX, wild-type A_β^b (ABB1), ABBΔ10, ABBΔ18, βCT1, and βCT2 transfectants were stimulated with Ag plus MAbs specific for endogenous E^k or transfected A_β^b molecules, and cAMP was extracted and measured as described in Experimental Procedures. The ratio of cAMP to A^b/E^k is given above each set of bars, and was calculated and normalized by dividing the pmol cAMP/2 × 10^5 cells following stimulation with Ag plus anti-A_β MAb (striped bars) by the pmol cAMP/2 × 10^5 cells following stimulation with Ag plus anti-E^k MAb (closed bars). cAMP values for cultures containing Ag alone (open bars) were subtracted from all values prior to the determination of ratios.

(B) Direct comparison of ratios of cAMP production, calculated as for (A), from a separate experiment.

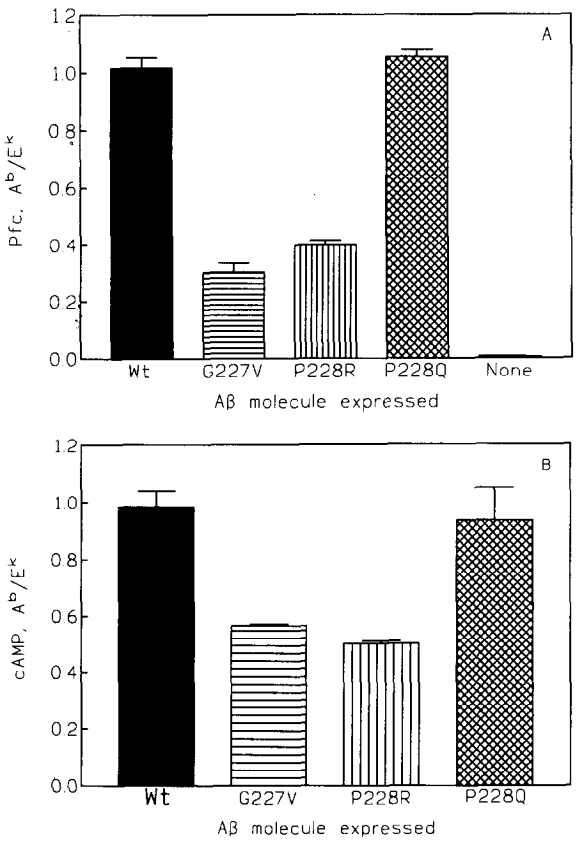


Figure 4. Induction of B Cell Differentiation and cAMP Generation via CY Domain Point Mutants of $A_\beta{}^b$

(A) pfc response of the mutants G227V, P228R, and P228Q compared with CH12.LX and ABB1. pfc ratios were determined as described for Figure 2; at least two independent subclones were tested for each mutant.

(B) cAMP induction via signaling through wild-type and point mutants of $A_\beta{}^b$. cAMP ratios were determined as described in Figure 3.

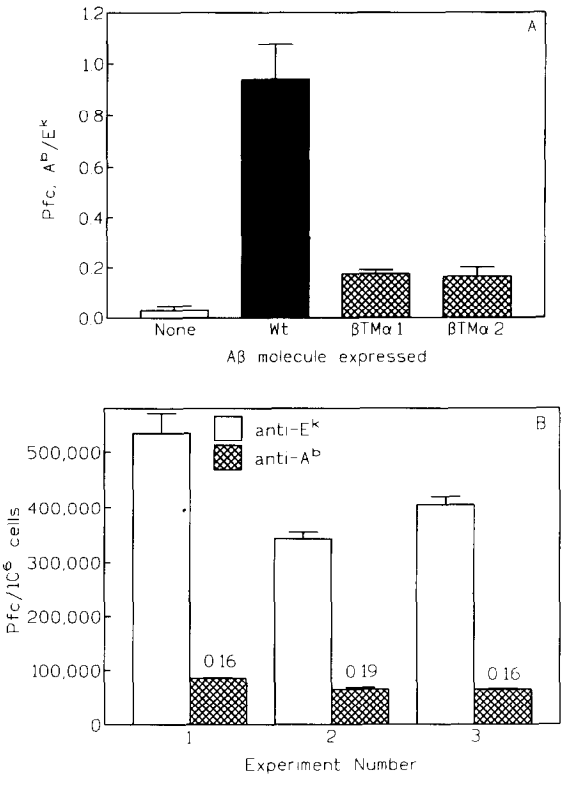


Figure 5. Class II-Mediated Signaling via TM Domain Mutants of $A_\beta{}^b$

(A) CH12.LX (none), wild-type $A_\beta{}^b$, and $A_\beta TM_\alpha$ transfectants (βTMα.1 and βTMα2) were stimulated with Ag plus MAbs specific for endogenous E^k or transfected $A_\beta{}^b$ molecules, and the ratio of Pfc to A^b/E^k was calculated as described for Figure 2.

(B) pfc values for three individual experiments performed on the subclone βTMα.2. Experiments were performed and ratios (values above cross-hatched bars) were calculated as for Figure 2A.

suggests that these residues either directly or indirectly interact with other proteins needed to increase intracellular cAMP. The repositioning, removal, or replacement of G227 and P228 (except for the allowable substitution of the Q residue at position 228) results in an inability to generate intracellular cAMP (Figures 3 and 4B), which correlates with the inability to deliver differentiative signals as determined by induction of IgM secretion (see Figures 1A, 2, 4A). The requirement for GP/Q in the correct position reinforces the close correlation between cAMP generation and induction of IgM secretion following class II signaling.

The Transmembrane Domain of $A_\beta{}^b$ Is Important for Class II-Mediated Differentiative Signals

Previously, we reported that a modest but consistent level of signaling was detectable following complete removal of the CY domain of A_β (Harton and Bishop, 1993; see Figure 1A), although this molecule (ABBΔ18) was unable to generate elevated intracellular cAMP (see Figure 3). This finding suggested the involvement of additional β chain domains in class II signaling. Consistent with this hypothesis, addition of dbcAMP in the presence of antigen, without direct ligation of class II, is insufficient to mimic differentiation signals delivered via wild-type class II (Bishop, 1991). However, near complete restoration of signaling was observed in the Δ18 mutant if class II was cross-linked in the presence of dbcAMP (Figure 5A, discussed below). Taken together, these observations strongly suggested that ligation of class II affects not only CY domain signaling, but that other regions of the class II molecule are important to the signal. To determine whether this contribution is dependent upon the TM domain of Aβ, we examined signaling via mutant Aβ molecules in which the TM domain of β was replaced with that of α ($A_\beta TM_\alpha$; see Figure 1B). The TM domain of α was chosen to replace β, as it was previously demonstrated that α TM domains successfully interact and bind to each other (Cosson and Bonifacino, 1992). Although membrane expression of this molecule was equal to that of wild-type $A_\beta{}^b$ transfectants (data not shown), and the molecule contains a wild-type CY domain, induced differentiation via $A_\beta TM_\alpha$ was markedly decreased (Figure 5). The substantial loss of function with this mutant (an 80%–85% decrease) demonstrates that the TM domain of the β chain is mediating a function crucial to signaling.

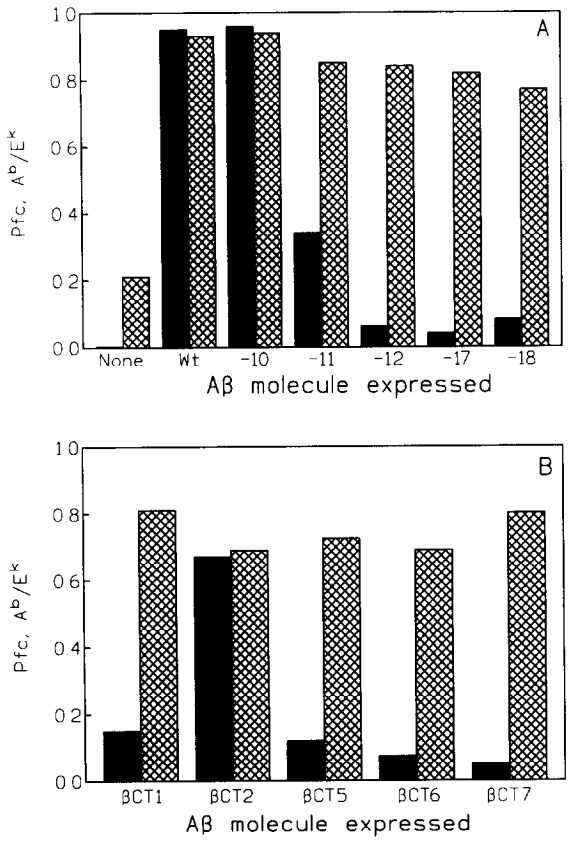


Figure 6. Enhancement by dbcAMP of Class II–Mediated Signals Generated by CY Domain Mutants of $A_\beta{}^b$

CH12.LX (none), wild-type $A_\beta{}^b$, ABBΔ10, Δ11, Δ12, Δ17, Δ18 (A) or βCT1, βCT2, βCT5, βCT6, and βCT7 (B) transfectants were stimulated with Ag plus MAbs specific for endogenous E^k or transfected $A_\beta{}^b$ molecules as in Figure 2, in the presence (hatched bars) or absence (solid bars) of the cAMP analog dbcAMP. The ratio of pfc to A^b/E^k was calculated as described for Figure 2. Data are representative of two experiments, which gave similar results.

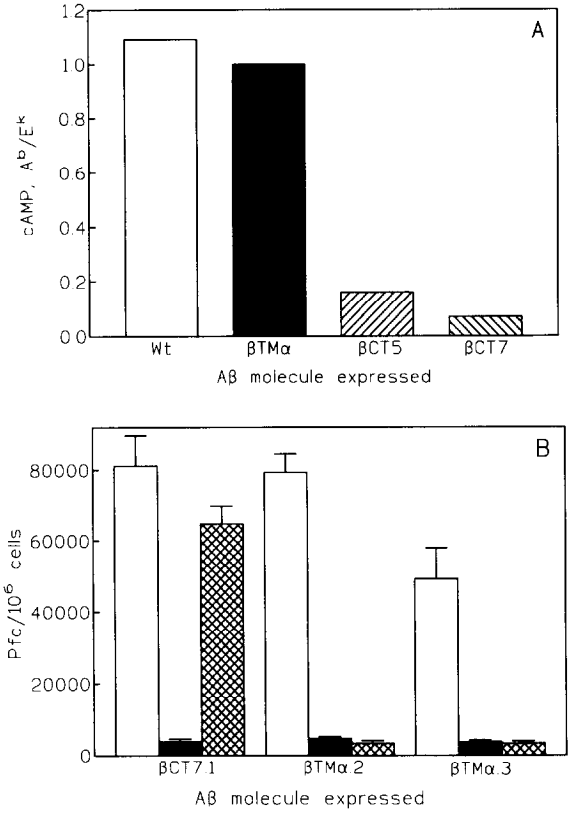


Figure 7. Mutation of the β Chain TM Domain Does Not Affect Class II–Mediated cAMP Generation

(A) Class II–mediated generation of intracellular cAMP in CH12.LX cells expressing wild-type $A_\beta{}^b$ or CY (βCT5 and βCT7) or TM (βTMα) domain mutants. Cells were stimulated with Ag plus E^k or A^b-specific MAbs, and cAMP quantitated as for Figure 3.

(B) A representative transfectant expressing the CY domain mutant βCT7 and two transfectants expressing the $A_\beta TM_\alpha$ mutant were cultured with Ag plus E^k or A^b-specific MAbs, plus or minus dbcAMP, as in Figure 6. pfc/10^6 viable recovered cells were determined as described in Experimental Procedures. Open bars, E^k-specific MAb; closed bars, A^b-specific Mab; cross-hatched bars, A^b-specific MAb plus dbcAMP. Results are mean ± SEM of triplicate cultures, and are representative of two similar experiments.

Direct Elevation of Intracellular cAMP Increases Signaling in Transfectants Expressing CY Domain Mutants of $A_\beta{}^b$

Because signaling-defective mutants of the A_β CY domain could not function in either MHC class II–mediated differentiation or cAMP elevation, it was of interest to determine whether elevating intracellular cAMP directly, together with class II engagement, could restore signaling in these mutants. Individual representative subclones expressing wild-type or CY mutant $A_\beta{}^b$ molecules were cultured with antigen and $A_\beta{}^b$-specific monoclonal antibodies (MAbs), with or without dbcAMP. Inclusion of dbcAMP significantly increased the effectiveness of signals in cells expressing signal-deficient class II molecules with CY domain mutations (Figure 6). Results shown for CH12.LX (bars labeled none) demonstrate, as reported previously (Bishop, 1991), that dbcAMP plus Ag alone, without class II engagement, stimulates only a modest amount of differentiation (2- to 3-fold above background). This amount is 10- to 50-fold lower than differentiation induced by anti-class II plus Ag. dbcAMP does not enhance signaling through endogenous E^k molecules (Bishop, 1991), or in transfected $A_\beta{}^b$ wild-type or ABBΔ10 (Figure 6A). Consistent with the observation that cAMP generation is maximal in cells transfected with βCT2, the addition of dbcAMP had no detectable effect on class II–mediated differentiative events in βCT2 mutants (Figure 6B). This further supports our conclusion that the major requirement of the cAMP pathway is G227 and P/Q228 in the appropriate location. In transfectants expressing mutant $A_\beta{}^b$ molecules that showed severely impaired signaling, however (Δ11, Δ12, Δ17, Δ18, βCT1, βCT4, βCT5, βCT6, βCT7), the combination of dbcAMP and ligation of $A_\beta{}^b$ increased signaling to approximately 80% of that seen via endogenous E^k (cross-hatched bars).

The TM Domain–Mediated Signal Is Distinct from the CY Domain–Mediated cAMP Response

To test the possibility that changes in the TM domain affected the ability of the molecule to generate cAMP fol-

lowing class II engagement, cAMP generation in cells expressing $A_\beta TM_\alpha$ was investigated. Ligation of $A_\beta TM_\alpha$ molecules led to an increase in intracellular cAMP comparable to that seen with engagement of wild-type $A_\beta{}^b$ molecules (Figure 7A). This result contrasts with the lack of class II–mediated cAMP elevation seen in the signaling-defective CY domain mutants βCT5 and βCT7. Consistent with this finding, addition of dbcAMP did not rescue the defective signaling seen in $A_\beta TM_\alpha$ mutants, as it did in CY domain mutants (Figure 7B). Thus, signals generated via the TM domain are independent of events leading to cAMP generation, indicating that signaling via the CY and TM domains of class II use distinct pathways. These pathways must be at least partially cooperative, however, as disruption of either leads to loss of the majority of class II–induced B cell differentiation.

Discussion

There is an increasing body of data supporting the role of MHC class II molecules as signal receptors for B lymphocytes (recently reviewed by Scholl and Geha, 1994). A number of early events (Bishop, 1991; St. Pierre and Watts, 1991; Fuleihan et al., 1992; Newell et al., 1993; Marshall et al., 1994), as well as more distal effects (Palacios et al., 1983; Bishop and Haughton, 1986; Wade et al., 1989; Hamano et al., 1990; Bishop et al., 1993; Newell et al., 1993; Truman et al., 1994) have been described following engagement of class II molecules. The role of the β chain of the class II αβ heterodimer has received the greatest attention, as several studies suggest the predominance of the β chain in signaling events (Bishop and Frelinger, 1989; Wade et al., 1989). Truncation of the CY domain of the α chain also appears to affect successful antigen presentation to certain T cell clones (Wade et al., 1994), but whether or not this effect involves signal transduction via class II is unknown. The present study focuses on a defined region of the class II A_β CY domain involved in signal transduction, and also demonstrates an important role for the TM domain of A_β in class II signaling.

We extend our previous structure/function analyses by demonstrating that within the eight membrane-proximal residues of the CY domain of A_β necessary and sufficient for class II–mediated differentiative signaling, the residues at positions 227 and 228, independent of specific residues at positions 221–226, are sufficient for a substantial portion of class II–mediated differentiative signals. Additionally, when in the appropriate position, these residues are sufficient to mediate class II–induced intracellular cAMP responses, demonstrating that a major signaling function of the CY domain of A_β is the generation of a cAMP response. This conclusion is consistent with the observations of others that cAMP analogs restore certain class II–mediated signals to truncated class II molecules, such as successful antigen presentation and translocation of a cAMP-dependent kinase (Nabavi et al., 1989; Wade et al., 1989; St. Pierre and Watts, 1991).

The induction of cAMP following ligation of MHC class II molecules on mouse B cells has been demonstrated previously (Cambier et al., 1987; Takahama et al., 1989; Bishop, 1991; Newell et al., 1993). The class II–mediated cAMP response appears to be important for the induction of PKC translocation to the nucleus (Cambier et al., 1987), the induction of apoptosis in naive resting B cells (Newell et al., 1993), the ability of anti-class II MAbs to inhibit lipopolysaccharide or T cell–derived growth factor-stimulated proliferation of B cells (Takahama et al., 1989), and the stimulation of class II–mediated antigen-dependent differentiation in CH12.LX (Bishop, 1991). Our results demonstrate that the observed cAMP dependency of these events may be attributed to the function of specific residues in the cytoplasmic domain of the A_β chain.

The function of G227 and P228 is position dependent and likely requires G and P/Q specifically, as changes at these positions (βCT5, βCT6, G227V, and P228R) nearly abrogate both differentiative signaling as well as intracellular cAMP generation. Data showing that βCT5 and βCT6 have a more drastic loss of function while still possessing either G227 or P228 alone suggest that the requirement for G and P is stringent, and further suggests the possibility that residual signaling observed with the point mutants is due to the presence of the wild-type residues in positions 220–226. This is also consistent with the observation that βCT2 only restores 70% of the wild-type signal, suggesting that residues 220–226 are required for optimal signaling. These data are consistent with the possibility that the CY domain of A_β, and specifically G227 and P228, interacts with proteins that ultimately activate a G protein; this, in turn, can activate adenylate cyclase and leads to increased cAMP. However, our data do not establish that these residues themselves are crucial for protein–protein contact; they may instead assure that the proper conformation for such contact is maintained.

G227 is absolutely conserved in A and E molecules and is generally conserved in HLA-D gene products. P228 is absolutely conserved in A molecules, while in E molecules, glutamine is substituted for P at position 228. In HLA-D gene products, this position contains either glutamine (Q) or histidine (H) (Harton and Bishop, 1993). We speculate that position 228 may function as a specific interaction site for a signaling protein or for an adapter molecule. There is recent precedence for this type of interaction, as proline is reported to be an important residue in certain class I peptide-binding motifs (Sidney et al., 1995). Q (in mouse E molecules and HLA-DP) effectively substitutes for P (Figures 1 and 4). This is not entirely surprising, as HLA-D molecules are capable of mediating increases in intracellular cAMP (J. A. H. and G. A. B., unpublished data). Although the wild-type CY domain of A_β is proline rich, an interaction with an SH3 domain requiring only a single P residue (as in the case of βCT2), or no P residues (I-E and HLA-D), seems unlikely. G227 may function simply as a spacer, allowing P228 to be accessible to an associated protein via free rotation at this position, as changing this residue to an alanine (βCT1), which may lead to repulsion by hydrophobic residues, abrogates function.

Addition of dbcAMP substantially complements differentiative signaling via A_β molecules with detrimental muta-

tions in their CY domains. This supports the conclusion that mutations in the CY domain of A_β that affect signaling act primarily by disrupting the generation of cAMP. The simplest explanation of these results is that only the cAMP pathway is affected by alteration of the CY domain of A_β. Although this hypothesis is consistent with the data, additional effects cannot be excluded.

We also report here a direct demonstration that the TM domain of A_β is important to class II–mediated B cell signaling. Addition of dbcAMP in the presence of antigen, without class II ligation, results in a minimal differentiative response, suggesting that increases in cAMP mediated by the CY domain of A_β are insufficient for complete signaling. Thus, we predicted the existence of another signaling pathway. This was further supported by our previous observation that a small but clearly demonstrable and consistent differentiative signal occurs in cells expressing ABBΔ18 (complete removal of the CY domain of A_β) following class II engagement. To test the possible involvement of the TM domain, we replaced the TM domain of A_β with that of A_α ($A_\beta TM_\alpha$; Figure 1B). The $A_\beta TM_\alpha$ mutant signals poorly (only 10%–20% of wild type), demonstrating that this domain is required for wild-type signal transmission. The potential effects of altered TM domains on the expression, pairing, and conformation of class II molecules was considered before designing the $A_\beta TM_\alpha$ molecule. Cosson and Bonifacino (1992) demonstrated that substitution of class II TM domains with those of Tac antigen abrogates surface expression and alters the conformation of the class II external domains. Others have shown that changes in the extracellular domains of class II, which alter binding of conformation-sensitive MAbs, are detrimental to pairing, surface expression, or both (Braunstein and Germain, 1987; Buerstedde et al., 1988). Replacement of the TM domain of A_β with that of A_α requires dimerization of A_α domains for pairing and surface expression. The study of Cosson and Bonifacino (1992) predicts that this combination will be successful, because their model suggests direct interactions of glycine-rich TM interfaces are important for successful heterodimerization and correct conformation. The α TM domain provides glycine interfaces predicted for β, and maintains the apposition of a G and a F at two serial interface positions in the carboxyl portion of the heterodimeric TM domain (Cosson and Bonifacino, 1992; unpublished data). Consistent with these predictions, transfectants of CH12.LX express $A_\beta TM_\alpha$ molecules on the surface at levels similar to other transfected A_β molecules, suggesting no major pairing or conformational changes. The possibility of minor conformational alterations cannot be entirely excluded, as conformation-sensitive A^b-specific MAbs have not been reported.

The generation of cAMP following ligation of $A_\beta TM_\alpha$ was equivalent to that for wild-type class II molecules, indicating that the $A_\beta TM_\alpha$ molecule is not unable to deliver signals. In addition, these data show that the TM signal is distinct from the CY domain–dependent cAMP response. As predicted, addition of dbcAMP did not detectably enhance signaling through $A_\beta TM_\alpha$. This suggests that other associated proteins, separate from those interacting with the CY domain, are mediating crucial aspects of class II signaling, and that the MHC class II molecule is likely part of a multiprotein signaling complex, as is true of membrane immunoglobulin and the T cell receptor.

Morio et al. (1994) have demonstrated the involvement of the src-kinase family members lyn and fgr in staphylococcal superantigen-induced class II signaling in the human B lymphoma line Raji. PTK activation has also been described following superantigen engagement of class II molecules on human monocytes (Matsuyama et al., 1993; Morio et al., 1994). Our results demonstrating a signaling pathway involved in B cell differentiation distinct from the cAMP pathway are consistent with the involvement of a PTK. It is possible that signals transmitted by the TM domain are linked to one or more PTKs, as phosphotyrosine containing proteins have been detected in the B cell line K46 following class II ligation even in the absence of class II CY domains (André et al., 1994). We have recently observed that differentiative signaling by wild-type class II molecules is inhibited by the PTK inhibitor herbimycin A when added together with anti-class II MAb 18 hr after addition of antigen. This treatment abolished the residual signal observed with the CY domain mutant ABBΔ18 but did not further decrease signaling in the TM domain mutant $A_\beta TM_\alpha$, which has a wild-type CY domain (data not shown). These data are consistent with the hypothesis that the TM domain of A_β is involved in activating a PTK.

A summary of class II signaling events affected by structural alterations in either the CY or TM domain is shown in Table 1. A_β chains, which contain at least eight membrane-proximal amino acids, including G227 and P228, show wild-type levels of signaling as measured by cAMP elevation and induction of immunoglobulin secretion. Alteration or removal of either G227 or P/Q228 results in loss of both these measures of signaling. Differentiation can be restored by addition of the cAMP analog dbcAMP, but dbcAMP does not further elevate immunoglobulin secretion via $A_\beta{}^b$ molecules that already induce effective activation, such as ABBΔ10 and βCT2. Alteration of the TM domain of A_β also results in loss of class II–mediated immunoglobulin secretion, but cAMP elevation remains intact, and dbcAMP does not complement the signaling defect.

Table 1. Summary of Structure–Function Requirements of CY and TM Class II Signaling

CY domain[a]	TM domain[b]	↑cAMP[c]	↑Pfc[d]	dbcAMP→↑Pfc[e]
+	+	+	+	–
–	+	–	–	+
+	–	+	–	–

[a] Class II β chain CY domain consisting of at least 8 membrane-proximal amino acids, including G^{227} and P or Q^{228} (wild type, ABBΔ10, βCT2, and P228Q).
[b] Wild-type β chain TM domain amino acid sequence.
[c] Engagement of class II results in elevation of intracellular cAMP.
[d] Engagement of class II in the presence of Ag results in induction of Ag-specific IgM secretion, measured as direct pfc.
[e] Addition of dbcAMP substantially increases Ag plus anti-class II–mediated IgM secretion, measured as direct pfc.

Experimental Procedures

Construction of Truncation Mutants

The CY domain truncation mutants of A_β^b (Δ10, Δ11, Δ12, Δ17, Δ18) and the replacement mutant βCT1 (Figure 1) have been described previously (Harton and Bishop, 1993). Replacement mutants of the CY domain of A_β^b (βCT2, βCT4, βCT5, βCT6, and βCT7) were generated by oligonucleotide-directed mutagenesis of the βCT1 replacement mutant using the polymerase chain reaction (PCR; Perkin Elmer-Cetus, Norwalk, Connecticut) essentially as described (Harton and Bishop, 1993). The $A_\beta TM_\alpha$ mutant was constructed with a combination of the megaprimer and overlap extension methods (Ho et al., 1989; Sarkar and Sommer, 1990), using the following strategy. In brief, the upstream primer TMαCTβ (*ACCATCTTCATCATTCAAGGCCTG*CGTCACAGGAGTCAGAAAGGACCT) was designed to introduce 15 nt of the carboxy-terminal portion of the A_α^b transmembrane coding sequence, and was used in conjunction with the downstream primer JH12 (AAGGTACCGAGTCACTGCAGGAGCCCT) to amplify the CY domain from an A_β^b cDNA. The product of this reaction was denatured and used as a megaprimer with the upstream primer CPβTMα (CAGTCTGAGTCTGCCTGGAGCAAG*ACTGTGGTGTGTGCCCTGGGGTTGT*) to amplify the TM domain coding sequence of the A_α^b-containing plasmid pKAB (Bishop and Frelinger, 1989). The amplification product of primers JH11 (TGTGAATTCGAGATGGCTCTGCAGATC) and D197B$^+$ (CATCTTGCTCCAGGCAGACTC) was used with wild-type A_β^b cDNA as template, yielding the external domains of A_β^b. The products of these reaction were used in an overlap–extension reaction, as previously described (Ho et al., 1989). Following the final amplification reaction, the product was digested with EcoRI and KpnI and cloned into these sites in the eukaryotic expression vector pcDL–SRa296 (Takebe et al., 1988). For each replacement mutant, the membrane proximal arginine anchor was left intact, as we predicted that the loss of a charged residue at this position might permit the CY domain to remain in the hydrophobic core of the plasma membrane. The G → V, P → R, and P → Q mutations in the minimal length CY domain were obtained from a saturation point mutant library of the Δ10 truncated A_β^b sequence. The generation of this library will be described in detail elsewhere (A. E. V., J. A. H., and G. A. B., unpublished data).

DNA Sequencing

Templates were prepared by alkaline lysis followed by purification on a Qiagen column (Studio City, California) according to the protocol of the manufacturer, or by miniprep as described (Maniatis et al., 1982; Hockenbery et al., 1990). Sequencing was performed as per the fmol cycle sequencing protocol (Promega, Madison, Wisconsin), with minor modifications, and samples were electrophoresed as described (Harton and Bishop, 1993). All templates were sequenced at least twice to ensure accuracy.

Cells

CH12.LX is a surface Ia^k, μ$^+$, δ$^+$, CD5$^+$ mouse B cell clone derived by single cell cloning of the CH12 lymphoma. The derivation and phenotype of CH12.LX have been previously described (Bishop and Haughton, 1986). The membrane and secreted immunoglobulin of CH12.LX is specific for the phosphatidylcholine moiety of sheep erythrocytes (SRBC) (Mercolino et al., 1986). Cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 300 μg/ml L-glutamine, and 0.01 mM 2-mercaptoethanol (BCM). Transfectants were selected and maintained in BCM containing 400 μg/ml of G418 (Sigma, St. Louis, Missouri).

MAbs

The MAbs used in this study were 34-5-3S (anti-$A_\beta^{b,d,p}$) (Ozato et al., 1982), and 14-4-4S (anti-E_α^k) (Ozato et al., 1980). All MAbs were prepared as hybridoma tissue culture supernatants in our laboratory. Immunoglobulin concentrations were determined by isotype-specific enzyme-linked immunosorbent assay, as described (Bishop and Haughton, 1987).

Transfection and Determination of Surface Expression

CH12.LX cells were transfected by electroporation as previously described (Bishop and Frelinger, 1989; Harton and Bishop, 1993) with 10–20 μg of A_β^b DNA, and 4 μg of a plasmid containing the selectable marker gene for *neo*^{R}, pSV2*neo* (Southern and Berg, 1982). Surface expression of the A_α^k/A_β^b heterodimer was confirmed for subclones expressing each truncation mutant by indirect immunofluorescence with the MAb 34-5-3S, followed by goat anti-mouse IgG–fluorescein isothiocyanate (Southern Biotechnology, Birmingham, Alabama). As a control, cells were stained with goat anti-mouse IgG–fluorescein isothiocyanate alone. This analysis was performed on an FACScan flow cytometer (Becton Dickinson, San Jose, California) using the 488 nm line from an argon–ion laser. Subclones bearing mutated A_β chains were selected for analysis only if they expressed those molecules at levels similar to those seen with transfected wild-type A_β^b, as described in our previous study (Harton and Bishop, 1993).

Assay for B Cell Differentiation

Induced differentiation of CH12.LX cells and transfectants was measured essentially as described (Bishop, 1991). In brief, cells were cultured in 0.2 ml of BCM in 96-well flat-bottomed tissue culture plates at a final concentration of 1.5 × 10^3 cells/well. Cultures containing antigen included a final concentration of 0.1% SRBC (Elmira Biologicals, Iowa City, Iowa). Cultures were incubated for 72 hr at 37°C in 5% CO_2 in air. All cultures were performed in triplicate. Direct hemolytic pfc were measured as described (Cunningham and Szenberg, 1968) and any background plaque formation (<5%) was subtracted to correct for basal levels of IgM secretion. Signaling by CH12.LX and transfectants is expressed in several of the figures as a ratio of the pfc value for cells cultured with Ag plus anti-A_β MAb divided by pfc value for cells cultured with Ag plus anti-E^k MAb. (Neither Ag nor class II alone induces pfc in CH12.LX; both are required; Bishop and Haughton, 1986). Thus, if signaling via transfected A_β^b is equal to signaling via endogenous E^k, the ratio will be close to 1. This is the case for signaling via wild-type A_β^b, as shown previously (Bishop and Frelinger, 1989; Harton and Bishop, 1993). For each transfectant, 3–4 individual clones were each tested several times. For those cultures containing the cAMP analog dbcAMP (Sigma Chemical, St. Louis, Missouri), dbcAMP was added at a final concentration of 3.0 mM, a concentration previously shown to be optimal for CH12.LX (Bishop, 1991).

Cyclic AMP Radioimmunoassays

Intracellular cAMP was extracted from stimulated or unstimulated CH12.LX and transfectants as previously described (Bishop, 1991). Intracellular cAMP concentrations were determined using a commercial RIA kit (NEN/DuPont, Boston, Massachusetts) with modifications allowing for miniaturization (Berg and Hornbeck, 1993). Data are expressed as the ratio of intracellular cAMP following ligation of wild-type or mutant A_β^b molecules/intracellular cAMP following ligation of the endogenous E^k molecule. As shown previously (Bishop, 1991), and also shown in Figure 3A, we typically see background cAMP amounts of 2–10 pmol/2 × 10^5 cells, rising to 10–35 pmol/2 × 10^5 cells following stimulation of a signaling-effective molecule. Fold stimulations are typically 2- to 5-fold; if a positive control (anti-E) stimulation is less than 2-fold, we do not consider this a successful stimulation, and do not attempt to calculate ratios from such data (the experiment would be redone).

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